Molecular Plant
Research Report

CellPress
Partner Journal

N-terminal truncated RHT-1 proteins generated by translational reinitiation cause semi-dwarfing of wheat Green Revolution alleles

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https://doi.org/10.1016/j.molp.2021.01.002

ABSTRACT

The unprecedented wheat yield increases during the Green Revolution were achieved through the introduction of the Reduced height (*Rht*)-*B1b* and *Rht-D1b* semi-dwarfing alleles. These *Rht-1* alleles encode growth-repressing *DELLA* genes containing a stop codon within their open reading frame that confers gibberellin (GA)-insensitive semi-dwarfism. In this study, we successfully took the hurdle of detecting wild-type RHT-1 proteins in different wheat organs and confirmed their degradation in response to GAs. We further demonstrated that *Rht-B1b* and *Rht-D1b* produce N-terminal truncated proteins through translational reinitiation. Expression of these N-terminal truncated proteins in transgenic lines and in *Rht-D1c*, an allele containing multiple *Rht-D1b* copies, demonstrated their ability to cause strong dwarfism, resulting from their insensitivity to GA-mediated degradation. N-terminal truncated proteins were detected in spikes and nodes, but not in the aleurone layers. Since *Rht-B1b* and *Rht-D1b* alleles cause dwarfism but have wild-type dormancy, this finding suggests that tissue-specific differences in translational reinitiation may explain why the *Rht-1* alleles reduce plant height without affecting dormancy. Taken together, our findings not only reveal the molecular mechanism underlying the Green Revolution but also demonstrate that translational reinitiation in the main open reading frame occurs in plants.

Key words: gibberellin (GA) signaling, DELLA, *Rht-1* dwarfing alleles, Green Revolution, wheat, translational reinitiation

Van De Velde K., Thomas S.G., Heyse F., Kaspar R., Van Der Straeten D., and Rohde A. (2021). N-terminal truncated RHT-1 proteins generated by translational reinitiation cause semi-dwarfing of wheat Green Revolution alleles. Mol. Plant. **14**, 679–687.

INTRODUCTION

During the Green Revolution in the 1960s, the benefits of intensified agronomic practices to dramatically increase cereal grain yields could only be fully achieved in combination with varieties containing semi-dwarfing alleles (Hedden, 2003). In wheat, the Green Revolution *Reduced height-1* (*Rht-1*) alleles were introduced to reduce plant height (Figure 1A), providing improved lodging resistance and allowing the application of higher fertilizer rates to substantially increase grain yields (Youssefian et al., 1992; Hedden, 2003). Despite the detailed study and exploitation of the growth effects of the *Rht-1* alleles since the Green Revolution and the characterization of the mutant genes just before the turn of the century (Peng et al., 1999), the proteins produced by *Rht-B1b* and *Rht-D1b* have never been identified.

The *Rht-1* loci contain the wheat *DELLA* genes, master regulators of the gibberellin (GA) signaling pathway (Peng et al., 1999; Thomas et al., 2016). DELLA proteins are part of a large, plant-specific family of transcriptional regulators, named GRAS proteins (Pysh et al., 1999). In contrast to the highly conserved C-terminal GRAS domain, the N terminus is hypervariable among GRAS family members. The N terminus of DELLA

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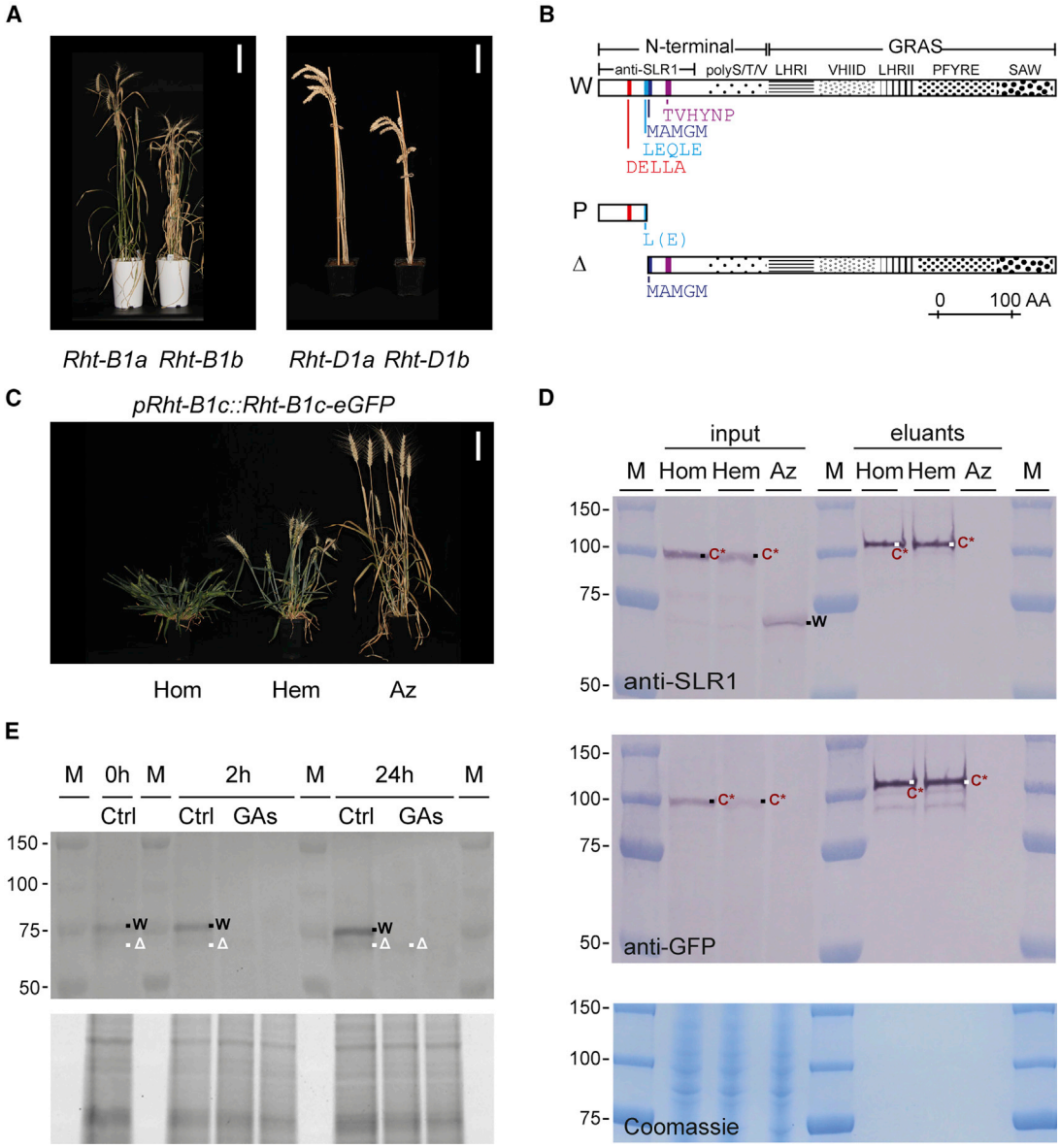

Figure 1. Immunodetection of RHT-1 proteins by anti-SLR1 *in planta*.

(A) Phenotypic effect of *Rht-B1b* in Maringá and *Rht-D1b* in Cadenza (bar, 10 cm).

(B) Schematic of wild-type RHT-1 protein (W) and two products expected to be produced by *Rht-B1b* or *Rht-D1b*: the 63- (including glutamic acid, E) or 62-AA peptide (P) and N-terminal truncated ΔN-RHT-B1 or ΔN-RHT-D1 (Δ), respectively. Conserved N-terminal motifs and GRAS subdomains are indicated, as well as the N-terminal region homologous to rice SLR1 against which the polyclonal anti-SLR1 antibody was raised (anti-SLR1). The GRAS domain contains two leucine heptad repeats (LHR I and LHR II), and the subdomains VHIID, PFYRE, and SAW. The N-terminal region is connected to the GRAS domain via the highly variable poly S/T/V domain.

(C) Phenotype of transgenic Fielder plants homozygous (Hom), hemizygous (Hem) and azygous (Az) for *pRht-B1c::Rht-B1c-eGFP*, segregating from one independent event (bar, 10 cm).

(D) Anti-SLR1 and anti-GFP immunodetection on crude protein (input) and eluants (after pull-down against eGFP) from developing spikes of transgenic Fielder plants shown in **(C)**. Wild-type RHT-1 (W) and GFP-tagged RHT-B1C (C*) indicated. RHT-B1C-eGFP appears slightly bigger in eluants, probably due to difference between extraction and elution buffers. Protein loading shown via Coomassie staining of input.

(E) Anti-SLR1 immunodetection on crude protein from developing spikes of Fielder wheat (*Rht-A1a*, *Rht-B1b*, *Rht-D1a*) incubated during indicated times in ultrapure water (Ctrl) or 10 μM GA_3 + 10 μM GA_4 (GAs). For GA treatments, two independent samples were loaded. Wild-type RHT-1 (W) and putative ΔN-RHT-B1 (Δ) signals indicated. Protein loading shown via fluorescent detection of all proteins before blotting (stain-free image below blot). M: protein size marker, sizes shown in kDa, predicted sizes in Supplemental Table 1.

proteins is characterized by the eponymous DELLA motif, followed by LEQLE and TVHYNP motifs (Figure 1B). In monocots, the LEQLE motif is directly followed by the MAMGM motif (Figure 1B) (Van De Velde et al., 2017). The DELLA and TVHYNP motifs are required for the interaction with the GA receptor, GIBBERELLIN INSENSITIVE DWARF1 (GID1), which

promotes the rapid degradation of DELLA proteins in the presence of bioactive GAs (Ueguchi-Tanaka et al., 2005; Murase et al., 2008).

In hexaploid bread wheat, DELLA proteins are encoded by three homeologous *Rht-1* genes, with the wild-type alleles designated as *Rht-A1a*, *Rht-B1a*, and *Rht-D1a* (Peng et al., 1999). The *Rht-1* height-reducing alleles result either from a mutation in *Rht-B1a* or *Rht-D1a* and are named *Rht-B1b* and *Rht-D1b* (previously *Rht1* and *Rht2*), respectively (Figure 1A) (Hedden, 2003). The mutations in *Rht-B1b* and *Rht-D1b* have only been found in wheat and comprise a C-to-T and G-to-T substitution of the first nucleotide in the codons encoding glutamine (Q, CAG; for *Rht-B1b*) and glutamic acid (E, GAG; for *Rht-D1b*) in the LEQLE motif, which in both cases results in an amber stop codon (TAG) (Figure 1B and Supplemental Figure 1) (Peng et al., 1999). Since the semi-dwarfism caused by *Rht-B1b* and *Rht-D1b* represents a clear gain-of-function phenotype, Peng et al. (1999) hypothesized that translational reinitiation at one or several of the AUG codons closely after the stop codons could generate an N-terminally truncated protein that confers the mutant phenotype (Δ in Figure 1B). An intragenic second-site suppressor mutation in *Rht-B1b* of durum wheat, located in the GRAS domain, and its phenotypic reversion from semi-dwarf to tall plants provided an indirect proof that the C-terminal part of the protein is indeed translated (Mo et al., 2018).

Here, we reveal the molecular mechanism underlying the function and phenotypes of the Green Revolution *Rht-1* alleles of wheat. Using transgenic and mutant lines, we demonstrate that the Green Revolution alleles encode N-terminal truncated DELLA proteins that are produced through translational reinitiation in the main open reading frame (ORF). Despite their low abundance due to the reduced efficiency of translation reinitiation, these N-terminal truncated proteins (Δ in Figure 1B), and not the N-terminal peptides (P in Figure 1B), cause dwarfing. In contrast to wild-type RHT-1, the N-terminal truncated protein is resistant to GA-mediated degradation and acts to constitutively repress GA responses, such as stem elongation. Taken together, the lower protein abundance and the GA insensitivity of the truncated protein explain the semi-dwarfing phenotype of *Rht-B1b* and *Rht-D1b*. Additionally, we show that translational reinitiation happens in stems and spikes but not in aleurone layers. This may explain the wild-type dormancy and GA response in the aleurone of *Rht-B1b* and *Rht-D1b*. Our findings set a new landmark for the engineering of the GA-signaling pathway in crop plants and, at a more fundamental level, for studying translational reinitiation in the main ORF in plants.

RESULTS AND DISCUSSION

Immunodetection of RHT-1 proteins and sensitivity to GA

Although immunodetection of DELLA proteins was successful in barley and rice using antibodies raised against the SLENDER1 proteins (i.e., anti-SLN1 and anti-SLR1, respectively; Gubler, 2002; Itoh et al., 2002), previous attempts to detect RHT-1 proteins in wheat were unsuccessful (Pearce et al., 2011). Considering the high sequence homology of rice and wheat DELLA proteins in the N-terminal regions against which the polyclonal anti-SLR1 antibody was raised (Supplemental Figure 1), we tested the cross-reactivity of anti-SLR1 toward RHT-1. Anti-SLR detected wild-type RHT-B1 when heterologously expressed in *Escherichia coli* (W* in Supplemental Figures 2B and 3). In wheat, a very faint signal of ∼75 kDa was detected in the leaf base (W in Supplemental Figure 4).

Next, we took advantage of the *Rht-B1c* allele that encodes a DELLA protein with a 30-amino acid (AA) insertion, which prevents its interaction with GID1 and subsequent GA-mediated degradation, resulting in RHT-B1C accumulation and extreme dwarfism (Figure 1C) (Pearce et al., 2011). RHT-B1C-eGFP was detected by both anti-SLR1 and anti-GFP when expressed under the native *Rht-B1c* promoter in homozygous and hemizygous spikes (C* in input in Figure 1D). Pull-down against eGFP enhanced these signals (C* in eluants in Figure 1D) and confirmed the specificity of anti-SLR1. In addition, anti-SLR1 detected endogenous RHT-1 in azygous spikes (W in Figure 1D), but these signals were barely visible in homozygous and hemizygous spikes. This absence is likely caused by the GA-signaling feedback mechanism (Hedden, 2016). The accumulation of GA-insensitive RHT-B1C proteins is expected to promote GA biosynthesis, signaling, and subsequent degradation of native RHT-1 proteins. Accordingly, bioactive gibberellins increased five- to 24-fold in *Rht-B1c* seedlings compared with wild type (Appleford and Lenton, 1991). Taken together, these findings demonstrate that anti-SLR1 can detect RHT-1 proteins in wheat leaves and spikes.

To confirm GA sensitivity of RHT-1 *in planta*, we treated wild-type Fielder spikes with GAs. RHT-1 proteins were rapidly degraded, thereby confirming their identity (W in Figure 1E). Since Fielder carries the wild-type *Rht-A1a* and *Rht-D1a* alleles, together with the mutant *Rht-B1b* allele, the 75-kDa signal in Figure 1E (W) is expected to correspond to GA-sensitive proteins produced by *Rht-A1a* and/or *Rht-D1a* (Supplemental Table 1). Intriguingly, faint signals at the predicted size of ΔN-RHT-B1 (Supplemental Table 1) were observed in some control and GA-treated samples (Δ in Figure 1E). Heterologous expression of *pT7::Rht-B1b-6xHN* in *E. coli* demonstrated that ΔN-RHT-B1 is generated from *Rht-B1b* and detected by anti-SLR1 (Δ* in Supplemental Figure 2B). The translation reinitiation site for ΔN-RHT-B1 generated in *E. coli* was determined at the first methionine following the stop codon in *Rht-B1b* (Supplemental Figure 3). This methionine is at the start of the MAMGM motif (Figure 1B), which represents the first five AAs of the SLR1-like1 (SLRL1) proteins, a group of GA-insensitive DGLLA proteins, specifically present in all monocots (Van De Velde et al., 2017). However, the signals of ΔN-RHT-B1 observed in Fielder were at the limit of detection and it was therefore not possible to draw firm conclusions on the production of ΔN-RHT-B1 by *Rht-B1b* (Δ in Figure 1E).

Rht-B1b and *Rht-D1b* produce an N-terminally truncated protein that causes dwarfism

To investigate whether N-terminal truncated proteins are produced by *Rht-B1b* and establish their phenotypic effect, transgenic lines were created expressing either the expected truncated protein fused to eGFP (*ΔN-RHT-B1-eGFP*) or a tagged version of the native *Rht-B1b* (*His-Rht-B1b-eGFP*), under the

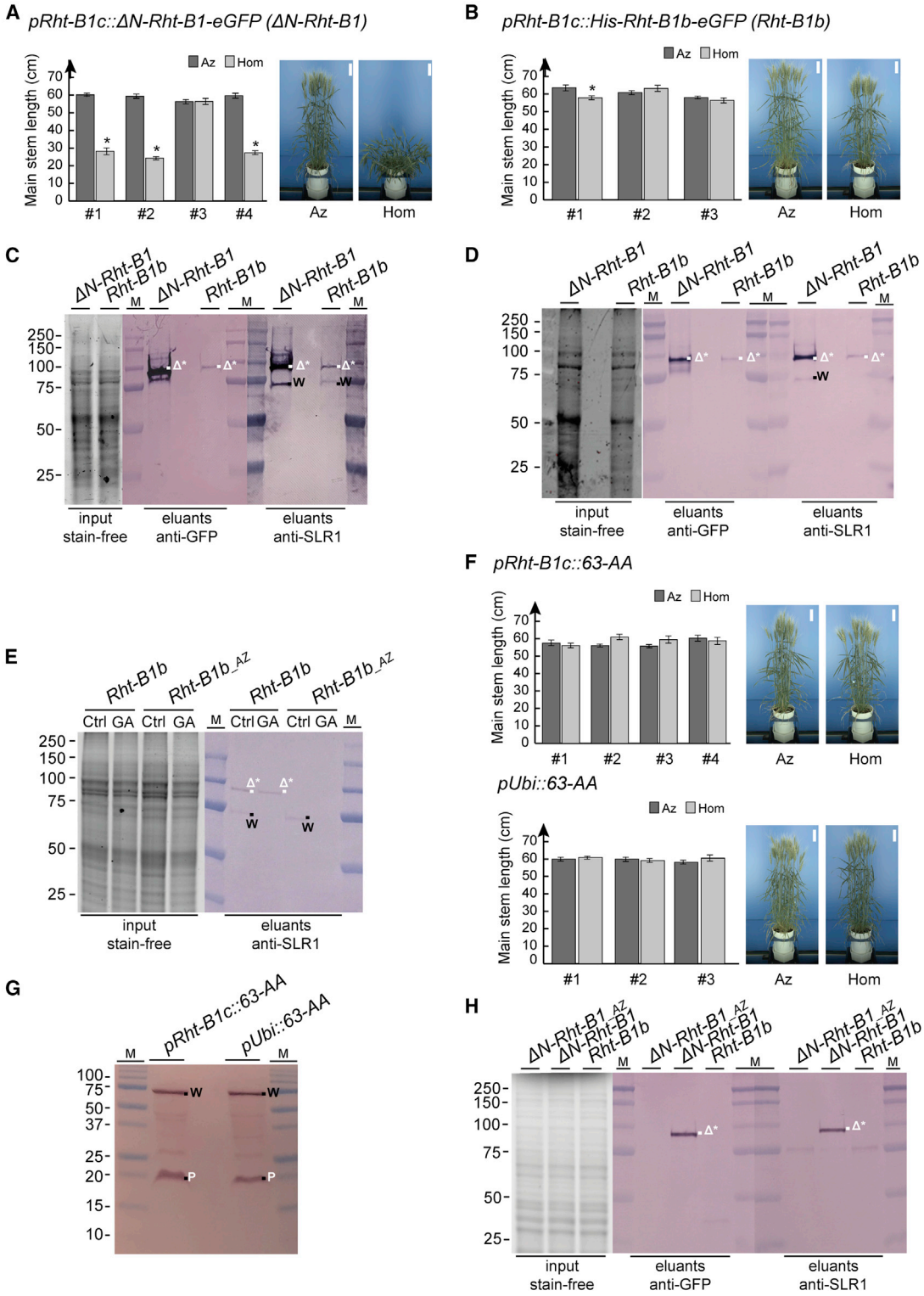

Figure 2. *Rht-B1b* produces an N-terminally truncated protein that reduces plant height.

Average main stem length ± SE of independent Proteo (*Rht-A1a*, *Rht-B1a*, *Rht-D1a*) events azygous (Az) or homozygous (Hom) for **(A)** *pRht-B1c::ΔN-RHT-B1-eGFP*, **(B)** *pRht-B1c::His-Rht-B1b-eGFP*, **(F)** *pRht-B1c::63-AA* and *pUbi::63-AA*; asterisk (*) indicates significant difference ($p < 0.01$, $N = 8$). Pictures show one representative homozygous and azygous plant of a statistically significant event if available (bar, 10 cm). Immunodetection in **(C)** spikes, **(D)** nodes, and **(H)** aleurone layers of *pRht-B1c::ΔN-Rht-B1-eGFP* (*ΔN-Rht-B1*) and *pRht-B1c::His-Rht-B1b-eGFP* (*Rht-B1b*) using anti-GFP or anti-SLR1 on eluants after pull-down against eGFP. For aleurone layers **(H)**, an azygous segregant of *pRht-B1c::ΔN-Rht-B1-eGFP* (*ΔN-RHT-B1_AZ*) was added as a negative control. Protein concentration of crude protein extract (input for pull-down) shown via fluorescent detection of all proteins (stain-free

(legend continued on next page)

native *Rht-B1c* promoter in Proteo (*Rht-A1a*, *Rht-B1a*, *Rht-D1a*). Three out of four homozygous *pRht-B1c::ΔN-RHT-B1-eGFP* T2 events showed a 35%–54% reduction of plant height relative to their respective azygous segregants (Figure 2A), demonstrating that the ΔN-RHT-B1 protein causes dwarfism. One out of three homozygous *pRht-B1c::His-Rht-B1b-eGFP* single-copy T2 events showed a significant height reduction of 9% compared with the segregating azygous plants (Figure 2B). In addition, one multicopy T2 event showed a significant reduction of 8% compared with the Proteo wild type and another multicopy event had several segregating plants showing extreme dwarfism (Supplemental Figures 5 and 6).

To further substantiate that the height reduction of *pRht-B1c::His-Rht-B1b-eGFP* is caused by the production of ΔN-RHT-B1, we performed protein pull-down using anti-GFP beads on crude protein extracts of young spikes and nodes of transgenic Proteo wheat plants expressing *pRht-B1c::ΔN-Rht-B1-eGFP* and *pRht-B1c::His-Rht-B1b-eGFP*. In these tissues and for both transgenic lines, anti-GFP and anti-SLR1 detected ΔN-RHT-B1-eGFP (Δ* in Figure 2C and 2D and Supplemental Figure 7), demonstrating that translational reinitiation occurs in *Rht-B1b-eGFP*, as suggested by Peng et al. (1999). Furthermore, GA treatment of *pRht-B1c::His-Rht-B1b-eGFP* spikes demonstrated that ΔN-RHT-B1 is GA insensitive (Figure 2E). The weaker signals of ΔN-RHT-B1 in *pRht-B1c::His-Rht-B1b-eGFP* compared with *pRht-B1c::ΔN-Rht-B1-eGFP* (Δ* for *Rht-B1b* in Figure 2C and 2D) suggest that production of ΔN-RHT-B1 by *Rht-B1b* is less efficient than from the truncated construct itself. This observation is consistent with a previous study demonstrating that translation is less efficient when occurring after reinitiation (Kozak, 2005). Although translational reinitiation was shown *in planta* from the start of main ORFs preceded by upstream open reading frames (uORFs) (Schepetilnikov and Ryabova, 2017; Gunišová et al., 2018), this is, to our knowledge, the first *in planta* evidence of translational reinitiation following a stop codon mutation within the main ORF. Taken together, the phenotypes and protein detection indicate that ΔN-RHT-B1 is generated by translational reinitiation in spikes and nodes of transgenic plants expressing *Rht-B1b* and is responsible for the reduction in stem elongation.

The additional band just below 75 kDa in spikes and *pRht-B1c::ΔN-Rht-B1-eGFP* nodes corresponds to the endogenous RHT-1 proteins (W in Figure 2C and 2D and Supplemental Figure 7). This finding suggests that endogenous RHT-1 proteins were co-immunoprecipitated with ΔN-RHT-B1-eGFP, probably as part of heterodimers between ΔN-RHT-B1 and wild-type RHT-1. In *Arabidopsis*, homodimerization of the REPRESSOR OF *ga1-3* (RGA) DELLA protein, as well as heterodimerization between RGA and GA-INSENSITIVE (GAI) DELLA proteins, occurs (Bai et al., 2012). Homodimerization was also shown for SLR1 and SLRL1 proteins in *Brachypodium* (Niu et al., 2019).

To exclude any direct or indirect effects of the N-terminal 63-AA peptide on plant height, it was expressed under the native *Rht-B1c* promoter or the constitutive maize Ubi promoter. Heterologous expression of *6xHN-Rht-B1b* in *E. coli* demonstrated that *Rht-B1b* produced the 63-AA peptide, and could be detected by anti-SLR1 (*P in Supplemental Figures 2B and 2C and 3, predicted size ~6 kDa). No differences in plant height were observed in 15 single-copy and 34 multicopy *pRht-B1c::63-AA* T0 events, nine single-copy and 37 multicopy *pUbi::63-AA* T0 events, or when comparing homozygous with azygous segregants in T2 plants (Figure 2F). Nevertheless, anti-SLR1 detected the 63-AA peptide in spikes of both *pRht-B1c::63-AA* and *pUbi::63-AA* (P in Figure 2G), together with endogenous DELLA proteins (W in Figure 2G). These findings strongly suggest that the 63-AA peptide has no effect on plant height.

Since *Rht-D1b* encodes an UAG one codon upstream of *Rht-B1b* (Figure 1B and Supplemental Figure 1), we investigated whether *Rht-D1b* also produced N-terminal truncated proteins (ΔN-RHT-D1) *in planta*. To this end, wheat near-isogenic lines (NILs) containing *Rht-D1a*, *Rht-D1c*, or *Rht-B1c* alleles in Cadenza were used (the wild-type contains the *Rht-1* alleles: *Rht-A1a*, *Rht-B1a*, and *Rht-D1a*). *Rht-D1c* is as dwarfed as *Rht-B1c* (Figure 3A) and derives from the presence of multiple copies of *Rht-D1b* at the *Rht-D1* locus (Pearce et al., 2011). Given the limited detectability of ΔN-RHT-B1 in Fielder (*Rht-B1b*) (Δ in Figure 1E), we hypothesized that the severe GA-insensitive phenotype of *Rht-D1c* would correlate with a higher abundance of N-terminal truncated DELLA proteins than in *Rht-B1b* or *Rht-D1b*. To distinguish between wild type (GA sensitive) and ΔN-RHT-D1 (GA insensitive), we treated young spikes and nodes with GA. *Rht-D1a* and *Rht-B1c*, respectively, serve as positive and negative controls for monitoring RHT-1 degradation in response to GA treatment. While the GA-insensitive RHT-B1C was not degraded in *Rht-B1c* mutants (C in Figure 3B and 3C), the RHT-1 signal was degraded by GA in the wild type (W in Figure 3B and 3C). The size of wild-type RHT-1 at ~75 kDa corresponds to that in Figure 1D and 1E, while RHT-B1C is slightly larger due to the 30-AA insertion (~80 kDa, Supplemental Table 1). As in homozygous and hemizygous *pRht-B1c::Rht-B1c-eGFP* spikes (Figure 1D), no wild-type signal was detected in *Rht-B1c* (Figure 3B and 3C), probably due to the GA-signaling feedback mechanism triggered by the accumulation of GA-insensitive RHT-B1C (Hedden, 2016).

The *Rht-D1c* spikes showed an equally strong signal just below the wild-type signal (Δ in Figure 3B). This signal was not degraded upon GA treatment (Δ in Figure 3B), which suggests that this band represents the ΔN-RHT-D1 protein that is insensitive to GA-mediated degradation. In *Rht-D1b* mutants, the same ΔN-RHT-D1 band could not be detected, probably due to the low protein abundance (Supplemental Figure 8). In nodes of *Rht-D1c*, both wild-type and ΔN-RHT-D1 signals were detected, albeit only after longer alkaline phosphatase reaction

imaging). **(E)** Anti-SLR1 immunodetection on eluants of homozygous (*Rht-B1b*) and azygous (*Rht-B1b_AZ*) spikes of *pRht-B1c::His-Rht-B1b-eGFP* treated with ultrapure water (Ctrl) or 100 μM GA_3 (GA) after pull-down against eGFP. **(G)** Immunostaining with anti-SLR1 on crude protein extract from spikes of *pRht-B1c::63-AA* and *pUbi::63-AA*. Wild-type RHT-1 (W), GFP-tagged ΔN-RHT-B1 (Δ*), and 63-AA peptide (P) indicated. M: protein size marker, sizes shown in kDa, predicted sizes in Supplemental Table 1. In spike eluants **(C)**, MS/MS confirmed both Δ* bands and W band of *pRht-B1c::ΔN-RHT-B1-eGFP* (Supplemental Figure 7).

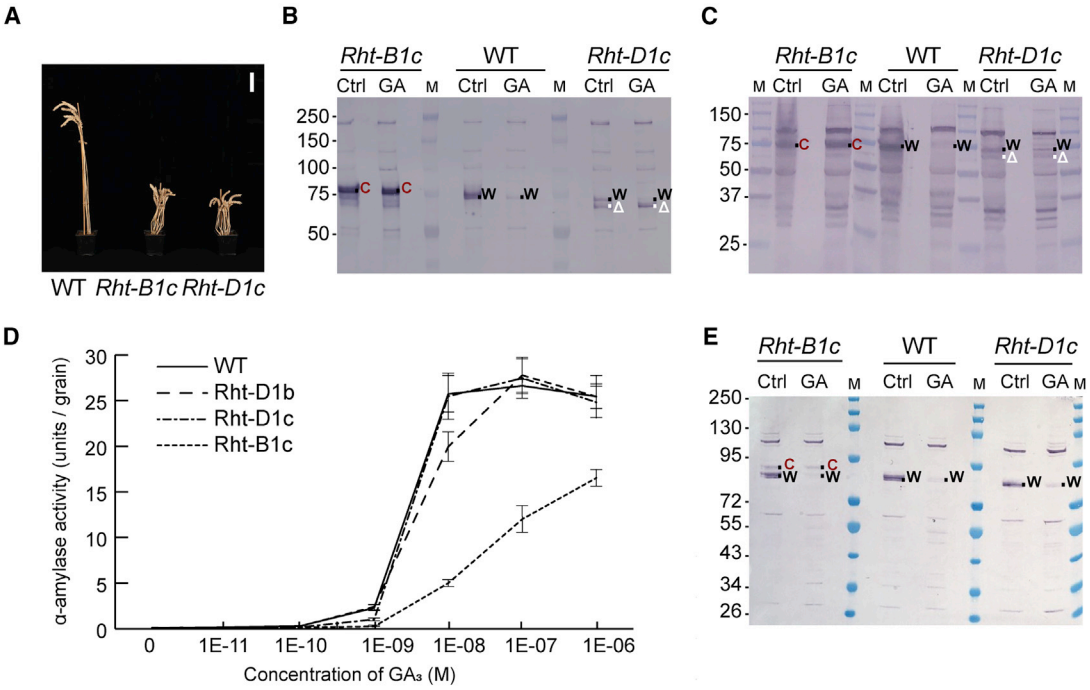

Figure 3. *Rht-D1c* produces an N-terminally truncated protein in spikes and nodes, but not in aleurone layers.
(A) Phenotypic effect of *Rht-B1c* (with *Rht-A1a*, *Rht-D1a*) and *Rht-D1c* (with *Rht-A1a*, *Rht-B1a*) NILs versus wild type (WT, *Rht-A1a*, *Rht-B1a*, *Rht-D1a*) in Cadenza. Immunodetection in crude protein extracts of **(B)** developing spikes, **(C)** developing nodes, and **(E)** aleurone layers; untreated control (Ctrl) or treated with 100 μM GA_3 (GA) for 4 h. RHT-B1C (C), wild-type (W), and ΔN-RHT-B1 (Δ) signals indicated. Non-specific signals serve as loading control. M: protein size marker, sizes shown in kDa, predicted sizes in Supplemental Table 1. **(D)** Average α-amylase activity ± SE in de-embryonated half-grains of wild type and NILs after incubation in different GA_3 concentrations during 72 h at 20°C (N = 5).

to visualize the signals (Δ in Figure 3C). Together, these findings strongly suggest that *Rht-D1b*, like *Rht-B1b*, produces N-terminal truncated DELLA proteins through translational reinitiation in young wheat spikes and nodes.

Truncated ΔN-RHT-B1 or ΔN-RHT-D1 are produced in nodes and spikes, but not in the aleurone layer

Previous research demonstrated that the GA signaling-mediated inhibitory effect of *Rht-B1b* and *Rht-D1b* observed on stem elongation is absent in grain dormancy (Gooding et al., 2012). More specifically, *Rht-B1b* and *Rht-D1b* reduce stem elongation but do not affect α-amylase production in the grain during germination (Gale and Marshall, 1973; Wu et al., 2011). This is in contrast with the phenotype of *Rht-B1c*, which has reduced GA sensitivity for both stem elongation and α-amylase production (Gale and Marshall, 1973; Wu et al., 2011), and is therefore more dormant than wild-type (Gooding et al., 2012; Derkx et al., 2017). *Rht-D1c*, also being severely dwarfed, does not exhibit the strong dormancy observed in *Rht-B1c* (Gooding et al., 2012). These findings suggest differences in the effects of *Rht-1* alleles in controlling GA responses in the wheat grain. To further investigate these differences, we compared GA-mediated induction of α-amylase activity in de-embryonated half-grains of wheat *Rht-1* NILs in Cadenza containing *Rht-D1b*, *Rht-D1c*, and *Rht-B1c* alleles. A dose-response assay was performed, measuring α-amylase activity after 72-h treatments using a broad range of GA_3 concentrations (Figure 3D). The increase in α-amylase activity stimulated by GA_3 was almost identical to wild-type Cadenza in both *Rht-D1b* and *Rht-D1c*, demonstrating a detectable increase in activity following treatment with 1 nM GA_3 and reaching a maximum at 10–100 nM. In contrast, *Rht-B1c* displayed reduced sensitivity to GA_3, with an increase in α-amylase activity only being detectable after a 10 nM GA_3 treatment, and the levels were markedly reduced compared with the other NILs at the higher GA_3 concentrations. These findings demonstrate that the *Rht-D1b* mutation, present in *Rht-D1b* and *Rht-D1c*, is not effective at inhibiting GA-mediated induction of α-amylase production in wheat aleurone cells and suggest clear differences in tissue-specific effects of *Rht-D1b* alleles. We performed immunostaining on crude extracts of control- and GA-treated aleurone layers of the two extremely dwarfed *Rht-B1c* and *Rht-D1c* alleles (Figure 3E). As observed in stems and spikes, *Rht-B1c* shows a signal at the expected size for RHT-B1C (Supplemental Table 1) that is insensitive to GAs (C in Figure 3E). In contrast to spikes and nodes, a GA-sensitive RHT-1 wild-type band was visible in *Rht-B1c* (W in Figure 3E), probably due to the absence of GA synthesis in the aleurone layer (Appleford and Lenton, 1997). Surprisingly, only one GA-sensitive band was detected in *Rht-D1c* (W in Figure 3E), indicative of the wild-type RHT-1 proteins (produced by *Rht-A1a* and *Rht-B1a*). The absence of the smaller, GA-insensitive ΔN-RHT-D1 protein, which was as abundant as wild-type RHT-1 in *Rht-D1c* spikes (Δ in Figure 3B), suggests that no ΔN-RHT-D1 protein is produced in the aleurone layer.

To substantiate this observation, we performed a pull-down assay using anti-GFP beads on crude protein extracts of

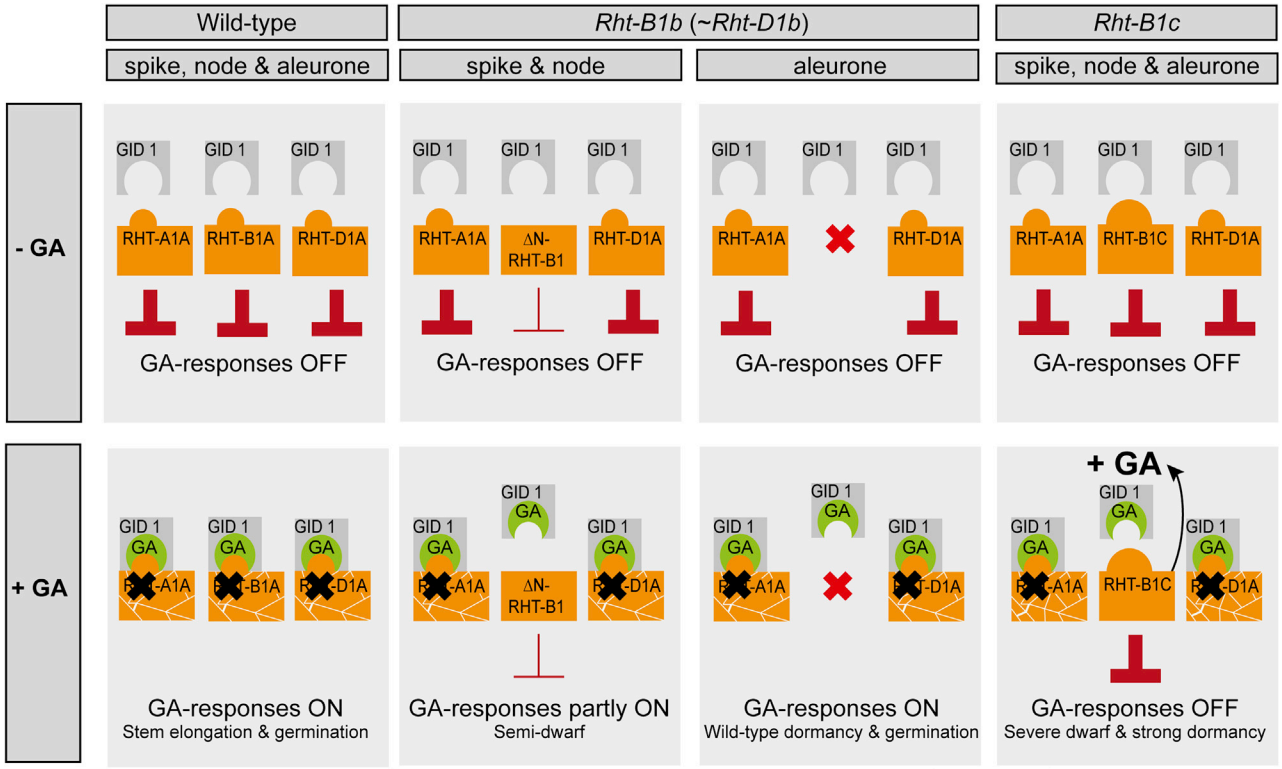

Figure 4. The proposed models showing DELLA-regulated responses in different tissues of hexaploid wheat carrying wild-type, *Rht-B1b*/*Rht-D1b*, or *Rht-B1c* allele.

Because signals remained below detection limit in the native *Rht-B1b* and *Rht-D1b*, the scenario is suggested based on GFP-tagged *Rht-B1b* (Figure 2) and *Rht-D1c*, which carries multiple copies of *Rht-D1b* (Figure 3). Hexaploid wheat contains three homeologous RHT-1 proteins (RHT-A1A, RHT-B1A, RHT-D1A), which are degraded by the 26S proteasome upon binding with the GA-activated GID1 receptor (signified by black cross on scattered RHT-1 protein). This degradation releases the repression of downstream GA-responses, such as stem elongation and germination. ΔN-RHT-B1 and ΔN-RHT-D1 are, respectively, produced by *Rht-B1b* and *Rht-D1b* in spikes and stem nodes (only the *Rht-B1b* scenario is shown in this figure), and cannot bind to the GA-GID1 complex (Pearce et al., 2011). The low efficiency of translational reinitiation results in low amounts of ΔN-RHT-B1 and ΔN-RHT-D1, which results in a weak repression of GA-responses, such as stem elongation, explaining the semi-dwarf phenotype. Since no ΔN-RHT-B1 or ΔN-RHT-D1 is produced in the aleurone layers (signified by red cross), the GA response of *Rht-B1b* and *Rht-D1b* in grains is identical to the wild type. *Rht-B1c* cannot interact with the GA-GID1 complex due to the 30-AA insertion (Pearce et al., 2011). This leads to a strong accumulation of RHT-B1C proteins, causing a stronger repression of GA-responses, such as stem elongation and germination, than in wild type or *Rht-B1b*/*Rht-D1b*. In addition, the feedback mechanism boosts GA synthesis (upward arrow toward GA), which results in a stronger degradation of RHT-A1A and RHT-D1A than in the wild type.

aleurone layers of transgenic Proteo plants expressing *pRht-B1c::ΔN-Rht-B1-eGFP* and *pRht-B1c::His-Rht-B1b-eGFP*, as well as on an azygous segregant of *pRht-B1c::ΔN-Rht-B1-eGFP* as a negative control. While ΔN-RHT-B1-eGFP was clearly detected in aleurone layers of *pRht-B1c::ΔN-Rht-B1-eGFP* (Δ* in Figure 2H), ΔN-RHT-B1-eGFP was not detected in *pRht-B1c::His-Rht-B1b-eGFP* (Figure 2H). This finding demonstrates that translational reinitiation of *Rht-B1b* is significantly reduced or absent in the aleurone layer and may explain why *Rht-B1b* and *Rht-D1b* have no altered responses during dormancy and germination. Therefore, the unique stop codon mutation followed by the MAMGM motif in *Rht-B1b* is required but not sufficient for translational reinitiation in all tissues.

In conclusion, translational reinitiation generates a truncated DELLA protein in stems and spikes of the gain-of-function alleles *Rht-B1b* and *Rht-D1b*, but not in the aleurone layer of seeds. Hence, the phenotypic effects of the Green Revolution alleles are caused by a truncated, GA-insensitive protein, whose translation depends on tissue-specific translational reinitiation (Figure 4). This finding represents a fundamental advancement in our understanding of the molecular basis of the Green Revolution alleles: they constitute unique gain-of-function alleles that enabled a specific regulatory circuit, recruited to establish the favorable semi-dwarfism (Figure 4).

METHODS

Plant material

Wheat NILs containing *Rht-D1b*, *Rht-D1c*, or *Rht-B1c* alleles in Cadenza were used in this study. The *Rht-D1c* and *Rht-B1c* alleles were introduced from Mercia (Pearce et al., 2011). The *Rht-D1b* allele was transferred from Avalon (Griffiths et al., 2009). The alleles were selected from homozygous progenies after six backcrosses to cultivar Cadenza (which contains the wild-type *Rht-1* alleles: *Rht-A1a*, *Rht-B1a*, and *Rht-D1a*) with recurrent selection for the dwarfing mutation.

Plasmid constructs and plant transformation

For details of all plasmid constructs, see the Supplemental information. Immature embryos of the spring wheat cultivars Proteo and Fielder were

transformed by cocultivation with *Agrobacterium tumefaciens*, according to the method described in Ishida et al. (2015). In each T0 event, the number of transgenes was determined by PCR of the *bar* gene. After selfing, this method was used for determining zygosity of independent T1 events.

Plant growth conditions, phenotypic measurements, GA treatments, and sampling

Plants were grown in controlled environments, as detailed in the Supplemental information. For height measurements, plants were grown in a randomized design to adjust for spatial effects. The main stems were marked at spike emergence (Zadoks 50) and their length was measured after flowering (Zadoks 80), from the soil level to below the spike. Developing spikes of approximately 1–2 cm and nodes were sampled at Zadoks 31 (Zadoks et al., 1974). Aleurone isolation was performed using mature wheat grains that were de-embryonated and of which the grain brush was removed. Sterilized half-grains were imbibed in 20 mM $CaCl_2$ solution at 20°C for 72 h in the dark, after which the endosperm was removed.

For treatments with GA_3 and/or GA_4, developing spikes, developing nodes, or aleurone layers of mature grains were completely submerged in 2 ml of treatment solution and were rotated during incubation.

Protein extraction and gel blot analysis

Total protein of different tissues was extracted and separated by SDS-PAGE. For additional details, see the Supplemental information. Subsequently, proteins were visualized by fluorescent detection after photo-activation (Criterion Stain Free gel imaging system, Bio-Rad). For immunostaining, proteins were transferred to a polyvinylidene fluoride membrane via semi-dry blotting using either a Trans-Blot Turbo (Bio-Rad) for spikes and nodes or iBlot system (Invitrogen) for aleurone extracted proteins. The blots were incubated overnight with monoclonal anti-GFP (3H9, 1/1000 dilution; ChromoTek GmbH) or polyclonal anti-SLR1 (1/5000 dilution; Cosmo Bio Co, Tokyo, Japan), followed by incubation with the appropriate alkaline phosphatase-conjugated secondary antibody. Detection of alkaline phosphatase reaction was performed according to the instruction manual until color development was complete (Bio-Rad), which could take 18–40 h.

Pull-down

After protein extraction, co-immunoprecipitation was performed with 90-μm agarose beads coated with monoclonal anti-GFP according to the instructions of the manufacturer (GFP-Trap_A, ChromoTek GmbH).

Measurement of α-amylase activity in wheat half-grains

Mature grains of Cadenza *Rht-1* NILs were harvested at the same time from greenhouse-grown plants. Hand-threshed grains were stored at 6°C and 80% humidity for approximately 6 months. Grains of equivalent size were selected based on weight. Five sterilized half-grains were incubated in the dark with agitation (50 rpm) in McCartney bottles containing 0.5 ml of 20 mM $CaCl_2$, 2% (v/v) Plant Preservative Mixture (Plant Cell Technology). Half-grains were homogenized, clarified by centrifugation, and the supernatant assayed for α-amylase activity using the Megazyme (Amylazyme) procedure. Units of activity (one unit releasing 1 mg of maltose from starch in 3 min at pH 6.9 at 20°C) were determined from a standard curve generated using purified barley malt α-amylase (Sigma).

Note added in proof

While this paper was submitted, additional proof for detection of RHT-1 proteins in E. coli and wheat was published by Smekenov et al. Journal of Genetic Engineering and Biotechnology (2020) 18:52. https://doi.org/10.1186/s43141-020-00072-4.

SUPPLEMENTAL INFORMATION

Supplemental Information is available at *Molecular Plant Online*.

FUNDING

D.V.D.S. acknowledges Ghent University for financial support. S.T. was supported by the Biotechnology and Biological Sciences Research Council Designing Future Wheat Cross Institute Strategic Programme (grant BB/P016855/1).

AUTHOR CONTRIBUTIONS

Conceptualization, K.V.D.V., A.R., D.V.D.S., and S.T.; investigation, K.V.D.V., F.H., S.T., and R.K.; formal analysis and data curation, K.V.D.V. and S.T.; writing — original draft, K.V.D.V.; writing — review and editing, A.R., S.T., and D.V.D.S.; funding acquisition, K.V.D.V, S.T. . and D.V.D.S.

ACKNOWLEDGMENTS

The authors are grateful to Peter Hedden (Laboratory of Growth Regulators, Olomouc, Czech Republic), David De Vleesschauwer and Ralf-Christian Schmidt (BASF Agricultural Solutions) for fruitful discussions. Peter Chandler (CSIRO, Canberra, Australia) provided grains of Maringá NILs. We wish to thank the Trait Research, Crop Genetics and Regulatory Science teams of BASF Agricultural Solutions (Ghent, Belgium) and the Rothamsted greenhouse and VCU staff for skilled technical assistance. K.V.D.V. was a recipient of a predoctoral fellowship, which was cofinanced by VLAIO (Brussels, Belgium) and Bayer CropScience. No conflict of interest declared.

Received: August 12, 2020
Revised: December 5, 2020
Accepted: January 5, 2021
Published: January 6, 2021

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